

THE ENUMERATION OF POLLUTION INDICATOR BACTERIA



Ontario

Ministry
of the
Environment

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DATED

DATES OF REVISION

The Enumeration of Pollution Indicator Bacteria
by Membrane Filter Procedures

Pollution indicator bacteria are used to determine the potability or potential health hazard associated with a water supply, bathing area or a wastewater effluent. Particular groups of pollution indicator bacteria were chosen because of their association with fecal material, which must always be considered as a potential source of disease-producing organisms. Under most conditions, these bacteria are present in numbers much greater than any disease-producing organisms and in general are considered to survive longer than pathogenic microorganisms. In addition, techniques for direct isolation and enumeration of pathogenic organisms have so far had limited success or acceptance by public health agencies.

Analyses for the detection and quantification of pollution in samples are most frequently performed for total coliforms, fecal coliforms and fecal streptococci. The interpretation and significance of these bacterial groups in water depends on the types and numbers of the organisms and the intended use of the water.

1. Sample Handling and Preservation

Bacteriological samples must be collected in Ministry of the Environment (MOE) sterilized glass or plastic bottles. The bacteriological examination of a water sample should be initiated immediately after collection. However, as this is seldom practical, more realistic arrangements must be established. The sample bottles should be chilled (not frozen) and transported to the laboratory within 24 hours. In any event, bacteriological analyses are not done on samples aged four days or more. For specific programs, samples must arrive at the laboratory within 24 hours. Samples containing chlorine as a disinfecting agent should be collected in sterile bottles to which sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$) has been added to neutralize any chlorine present.

2. Selection of Method

The two procedures commonly used to detect and quantify pollution indicator bacteria are the Most Probable Number (MPN) method and the Membrane Filter (MF) method. Each method has its own advantages and disadvantages, but for the most part they are considered to give comparable results. From a statistical aspect, the MF method gives direct counts; better reproducibility of results; produces results in 24-48 hours, and allows the examination of larger volumes of sample than the MPN method. The examination of water samples with high turbidity due to algae or other material may not permit testing of sufficient

samples with low numbers of indicator organisms relative to high numbers of other organisms or the presence of interfering substances may require the use of the MPN procedure to obtain accurate results.

TOTAL COLIFORM AND BACKGROUND COUNTS
MEMBRANE FILTER ANALYSIS

SUMMARY	
Organisms Determined	The test is designed to measure total coliform and non-coliform or background bacteria present in water samples from a variety of sources.
Interpretation of Results	In drinking water samples, the presence of total coliform bacteria indicates inadequate treatment of the water. Background bacteria represent non-coliform bacteria, which if present in large numbers may indicate poor quality water; they may also interfere with the determination of total coliforms. For more information consult the MOE's "Drinking Water Objectives" and "Guidelines and Criteria for Water Quality Management in Ontario".
Principle of Method	Each bacterial cell deposited on the membrane filter and given a suitable nutrient source has the potential to grow and multiply sufficiently to form a bacterial colony with distinguishing characteristics to permit a differential count being made.
Time required for Analysis	Depending on the density of bacteria in the sample, and hence the number of dilutions required to obtain plates suitable for counting, the analysis time for one sample would range from 5-10 minutes. Incubation time for the total coliforms is 22-24 hours.

Range of Application	Maximum sample volume for which an analysis is usually done is 100 ml. The upper limit of bacteria determined is dependent only on the number of dilutions required to obtain a suitable count.
Standard Deviation	Performance characteristics are not yet available.
Accuracy	Performance characteristics are not yet available.
Limit of Detection	Performance characteristics are not yet available.
Shortcomings and Interference	Improper choice of dilutions for membrane filters will affect the ability to obtain plates suitable for counting. High populations of non-coliform bacteria and toxic substances in the sample or membrane filters will interfere with colony and sheen development of coliform bacteria.
Minimum Volume of Sample	150-160 ml of sample is preferred when tests for several groups of bacteria are requested.
Preservation and Sample Container	MOE 6 oz sterile glass bottles should be used for collection of samples for drinking water analyses. Those from distribution systems should contain sodium thiosulphate to neutralize any chlorine residual. Samples should be iced if possible during transportation to the laboratory and arrive within 24 hours of the sampling time.

Safety Considerations

Bacteriological samples, particularly those from sources of water suspected of pollution, may contain pathogenic organisms, and should be handled in a manner to prevent contamination of the sampler and the analyst.

FECAL COLIFORM AND FECAL STREPTOCOCCUS COUNT
MEMBRANE FILTER ANALYSIS

SUMMARY	
Organisms Determined	The fecal coliform and fecal streptococci tests are frequently done conjointly to determine any interrelationship (iii).
Interpretation of Results	Fecal coliforms and fecal streptococci should be absent from potable drinking water samples. In natural waters, use is made of fecal coliform (FC) to fecal streptococci (FS) ratios of counts to determine the nature of the pollution. FC:FS ratios greater than 4.0 suggest human fecal pollution whereas FC:FS ratios less than 0.7 suggest animal fecal pollution. Intermediate ratios suggest a mixture of human and animal fecal pollution, but they may only indicate a more rapid decrease in viability of one population of organisms over another. The FC:FS ratio can only be used when both indicators suggest polluted conditions (FC > 100/100 ml or FS > 20/100 ml) and is applicable only to results from areas in the immediate vicinity of the waste effluent discharge. For more information consult the MOE's "Guidelines and Criteria for Water Quality Management in Ontario".
Principle of Method	Each bacterial cell deposited on the membrane filter and given a suitable nutrient source has the potential to grow and multiply sufficiently to form a bacterial colony with distinguishing characteristics to permit a differential count being made.

Time required for Analysis	Depending on the density of bacteria in the sample, and hence the number of dilutions required to obtain plates suitable for counting, the analysis time for one sample would be about five minutes for each parameter. Incubation time for fecal coliforms is 20- 1 hour and for fecal streptococci, it is 48- 3 hours.
Range of Application	Maximum sample volume for which an analysis is usually done is 100 ml. The minimum bacterial count is one organism per 100 ml. The upper limit of bacteria determined is dependent only on the number of dilutions required to obtain a suitable count.
Standard Deviation	Performance characteristics are not yet available.
Accuracy	Performance characteristics are not yet available.
Limits of Detection	Performance characteristics are not yet available.
Shortcomings and Interference	Improper choice of dilutions for membrane filters will affect the ability to obtain plates suitable for counting. High populations of organisms, inhibitory factors associated with membrane filters and/or toxic substances in the samples will interfere with the development of the organism for which the test was designed.
Minimum Volume of Sample	150-160 ml of sample is preferred when tests for several groups of bacteria are requested.

Preservation and Sample
Container

MOE 6 oz sterile glass bottles or sterile 250 ml Nalgene bottles may be used for sample collection. Sample bottles for collection of chlorinated waters should have sodium thio-sulphate added. All samples should be iced during transportation to the laboratory and preferably arrive for analysis within 24 hours of sampling time.

Safety Considerations

Bacteriological samples, particularly those from sources of water suspected of pollution, may contain pathogenic organisms, and should be handled in a manner to prevent contamination of the sampler and the analyst.

Pollution Indicator Bacteria
Membrane Filter Analysis

1. Introduction

The determination of total coliforms, fecal coliforms and fecal streptococci by membrane filtration employs similar procedures, differing only in the growth medium, incubation temperature and time, and the differentiation of colonies on MF petri plates. A general description of the membrane filter procedure will be given first and this will be followed by individual descriptions of salient factors to be observed for the analysis of total coliforms, fecal coliforms and fecal streptococci.

2. Interference and Shortcomings

The enumeration of each of the indicator bacteria depends on the selection of an appropriate volume of sample for testing purposes. In some instances, the sample volume may be too small to detect any indicator organisms. Conversely, if the sample volume is too large, an overgrowth or overabundance of bacterial colonies may interfere with obtaining an accurate count of the respective indicator group.

For certain samples, bacterial cells may have been exposed to adverse environmental factors which lower their probability for survival and growth on a membrane filter medium. Similarly certain membranes interfere with the growth of bacterial colonies (i, ii).

3. Apparatus

a) Sterile Equipment

- 1) pipettes - 1 or 2 ml for 1 ml deliveries
- 10 ml for 5 and 10 ml deliveries.
- 2) graduate cylinders - 100 ml for 100 ml volumes
- 50 ml for 50 ml and 25 ml volumes.
- 3) disposable petri dishes - round 50 x 12 mm
- square 100 x 15 mm.
- 4) 47 mm membrane filters with grid (0.45 pore size).
- 5) membrane filtration unit (glass or stainless steel) consisting of individually packaged funnel and base.
- 6) 47 mm absorbent pads (for broth media).

b) Non-sterile Equipment

- 1) two 50 ml beakers - one containing 20 ml of 95% ethanol for flaming forceps and one for pouring off excess broth media.
- 2) blunt-end forceps for handling membrane filters.
- 3) one polyethylene jar (2 litre) for used pipettes.
- 4) one 2 litre, 2 outlet vacuum Erlenmeyer (waste collection) flask with vacuum tubing and one-way valve connected to the bottom outlet.
- 5) one 1 litre, 2 outlet, plugged, vacuum Erlenmeyer (water trap) flask connected with vacuum tubing, from the bottom outlet to the 2 litre flask and from the top outlet to the vacuum source.
- 6) one polyethylene bucket for wastewater (or to drain).
- 7) one bunsen burner.
- 8) plastic "cakettes" for incubation of petri dishes.
- 9) one retort stand and clamp for holding membrane filter funnel when membrane filter is being removed from base. (optional).

c) Accessory Equipment

- 1) Waterbath and/or Incubators for maintaining temperatures at $\pm 0.5^{\circ}\text{C}$ in the ambient to 60°C range.
- 2) Stereoscopic microscope with 10X magnification for counting bacterial colonies.
- 3) Cool white fluorescent light for illuminating bacterial colonies.
- 4) Slanted (15°) wooden stage for resting petri dish during the counting operation.

4. Reagents

- a) Buffered Water - consists of 1000 ml of distilled water to which 1.25 ml of 0.25 M KH_2PO_4 (previously adjusted to pH 7.2 with 1N NaOH) is added. The buffered water is made up in the containers listed below and sterilized in an autoclave.
 - 1) 4 litre rinse bottle with dispensing nozzle.
 - 2) 99 ml dilution blanks.
 - 3) 90 ml dilution blanks.
- b) Disinfectant for swabbing benches, disinfecting pipettes and filter effluent:
 - 1) Wescodyne ("tamed iodine" - 1:30 with water).
 - 2) Dettol (1:5 with water).
- c) Bacteriological Media:
 - 1) Total Coliforms - Bacto m-Endo Agar LES (Difco).
 - 2) Fecal Coliforms - Oxoid MacConkey Membrane Broth.
 - 3) Fecal Streptococci - Bacto m-Enterococcus Agar (Difco) - preparation of the above media will be described fully in section (7).

5. A. Procedure - General

- a) Samples must be kept refrigerated before analysis. Only samples which can be analyzed within one hour should be kept on the work bench. All others should be kept under refrigeration until they are required for analysis.
- b) Preparation for membrane filtration:
 - 1) Sterile technique must be employed throughout the analysis procedure.
 - 2) The work bench area is swabbed thoroughly with the Dettol solution and wiped dry.
 - 3) Clean vacuum flasks and hoses are interconnected.
 - 4) A small amount of Wescodyne is poured into the waste bucket, pipette jar and waste collection flask.

- 5) The 4 litre rinse water bottle is set above the working area to allow for gravity flow. The dispensing hose and nozzle are hung using the retort stand and clamp above the water collection flask into which the membrane filter funnel and base will be placed. The hose may be fastened with tape to the clamp so that the flow of rinse water is not restricted and the glass nozzle does not come in contact with any other objects.
- 6) The samples are arranged in chronological order by laboratory number on the work bench or on the basis of priority.
- 7) Petri dishes are laid out opposite each sample. Those containing Endo agar LES and Enterococcus agar are inverted and the laboratory number and dilution are marked on the back of the plate.

Petri dishes for fecal coliform analysis are prepared at the time of analysis. Using sterile technique, 2 ml of MacConkey membrane broth are placed in each petri dish followed by an absorbent pad. For this analysis, the laboratory number and dilution are marked on the lid of the petri dish.

- 8) The membrane filter unit (funnel and base) is unpackaged and placed on the waste collection flask.

c) Preparation of dilutions:

- 1) Samples for microbiological analysis frequently have large numbers of organisms and require a series of one or more dilutions to provide a suitable distribution of colonies on the membrane filter before a satisfactory count can be made. Determination of the correct dilution series may be achieved by reference to previous analyses; knowledge of the nature of the sample; or sometimes based only on the observable turbidity of the sample and personal judgement.
- 2) Drinking water samples and relatively clean surface water samples usually require no dilution of the sample and aliquots from 100 to 1 ml are selected to give the appropriate distribution of organisms on the membrane filter. Samples from rivers, sewage effluents and other polluted sources frequently require one or more dilutions of the original sample (ten-fold dilution series is preferred).

- 3) Dilution of samples are made up in buffered water dilution blanks just prior to the membrane filtration procedure. They should not be left standing on the bench for more than 5-10 minutes before filtration takes place.
 - 4) The sample bottle is shaken 25 times vigorously.
 - 5) The cap on the sample bottle is held firmly by the fifth finger of one hand, while the bottle is unscrewed with the other hand held at the bottom of the bottle. The mouth of the bottle is flamed and one ml of sample is withdrawn with a pipette. The mouth of the sample bottle is flamed again and the cap screwed back into place.
 - 6) The one ml aliquot is then dispensed into a 99 ml dilution blank after first removing the cap as previously described. Before withdrawing aliquots for either further dilutions or filtration, the dilution blank must be shaken 25 times vigorously. If a ten ml aliquot is removed, this represents a 1:10 dilution of the original sample; if a one ml aliquot is removed, this is equivalent to 1:100 dilution. Higher dilutions are made by repeating the above operation. An alternative method of preparing a ten-fold dilution series may be done using 90 ml dilution blanks and 10 ml pipettes for transferring 10 ml aliquots into each dilution blank.
 - 7) Pipettes are used only once before discarding in the polyethylene jar.
- d) Membrane Filtration:
- 1) Prior to filtration of each sample or dilutions thereof, a control membrane is prepared. The vacuum supply of the membrane filter unit is turned on. The forceps are placed in the beaker containing 20 ml of 95% alcohol for several minutes; removed and flamed. Using the forceps, a membrane filter is removed from its package and held in one hand, while the membrane filter funnel is removed and held in the other hand. The membrane filter (grid side up) is then placed on the screen of the membrane filter base and the funnel is returned to the base and fastened securely. The forceps are returned to the alcohol beaker.

- 2) The funnel is then rinsed three times each with 20-30 ml of buffered water from the 4 litre rinse water bottle. The forceps are flamed again; the funnel removed and held; the membrane filter carefully removed from the base with the forceps while the funnel is replaced on its base.
- 3) The other hand picks up the half of the petri dish containing the medium and while holding it at an angle of 45° , the membrane filter is carefully rolled flat on one quadrant of the medium in the petri dish (grid side up). The lower portion of the petri dish is then inverted and placed back on its lid and the forceps are returned to the alcohol. Care must be exercised when placing the membrane filter on the medium that no air bubbles are entrapped which would impede the diffusion of nutrients into the membrane filter. The forceps may be gently used around the edge of the membrane to remove entrapped air, but never in the area of the filtration.
- 4) Filtration of the respective portions of the sample either from dilution blanks or the original sample proceeds in the manner essentially as described above. Volumes of sample from 100 ml to 25 ml are measured out into a sterile graduate cylinder. Volumes of 10 to 1 ml are pipetted for filtration. Volumes of less than 1 ml are not appropriate for analysis because of greater inaccuracies in pipetting small volumes. The first aliquot filtered is always the one containing the least amount of sample or the highest dilution of the sample.
- 5) Membrane filtration of an aliquot of sample proceeds as follows: a new membrane filter is positioned on the membrane filter base; the sample or dilution blank is shaken vigorously 25 times before measuring the volume for filtration; buffered rinse water is swirled into the funnel to a depth of $1/2$ " before and during dispensing of the aliquot (facilitates uniform distribution of cells on the membrane filters); the funnel is rinsed thoroughly 3 times each with 20-30 ml of buffered water; the membrane filter is removed from the unit and positioned on the growth medium in the petri dish. As before, sterile technique in handling the membrane filter with the forceps; the dispensing of the sample; and putting the membrane filter on the growth medium; must be carefully observed. Any accidental contamination of the membrane filter, pipettes, graduate cylinders, bottle caps, etc. will require discarding of the contaminated object and the operation begun again.

6) The positioning of a membrane filter on a petri dish containing a broth medium (e.g. MacConkey Broth) and pad should always precede the decanting of excess broth medium from the petri dish.

7) Item 5 above is repeated for each aliquot of sample filtered. Items 1-5 are repeated for each sample.

e) Incubation:

All petri dishes containing their respective membrane filters must be incubated within 30 minutes of the filtration step. The petri dishes are placed in separate plastic cakettes depending on the type of analysis, incubation period and incubation temperature. They are always incubated in an inverted position with the grid side of the membrane facing down. The cakettes are lined with moistened paper to provide a humid atmosphere. Each cakette is labelled to indicate the type of analysis, the date and time of incubation and the date and time of counting colonies on the membranes.

f) Counting:

1) At the end of the incubation period, the bacterial colonies growing on the membranes are counted using a stereoscopic microscope with 10X magnification. Each petri dish is positioned on the slanted base and a cool white fluorescent light is set up to provide the best illumination.

2) Petri dishes should be observed and counts made within the designated incubation period. Prolonged incubation or standing of plates before observations are made could give inaccurate results of some types of colonies, if colour differentiation is required for identification of a particular group of micro-organisms.

5. B. - Procedure - Total Coliform Analysis:

a) The m-Endo agar LES plates for the total coliform analysis are incubated at $35 \pm 0.5^{\circ} \text{C}$ for 22 ± 2 hours. The plates are counted immediately after removal from the incubator using the stereoscopic microscope at a magnification of 10X.

- b) All colonies with a dull to bright, metallic, green-gold sheen are considered members of the coliform group. The sheen may consist of only a central spot which may gradually increase in size to cover the entire colony. Colonies which lack sheen may be red, pink, blue, white or colourless and are considered as background colonies (non-coliforms). Doubtful colonies should be confirmed by transferring a small amount of inoculum from the colony to EC broth with incubation at 35°C for 24-48 hours. Gas production constitutes a positive result.
- c) An ideal density range of colonies on a membrane filter would consist of a total count not exceeding 300 colonies and a coliform count of 20-80 colonies. Plates with conditions unsuitable for counting would be: those with sheen colony counts greater than 150; plates showing confluent growth of colonies; control membranes with greater than 10 background colonies and/or one or more sheen colonies. Under these conditions, a repeat analysis should be made at a more appropriate dilution if the sample does not exceed the age limit or another sample should be requested.
- d) If the total count exceeds 300 colonies/plate, but a suitable coliform count is obtained, background counts may be estimated by counting colonies on 10 squares of the membrane filter and multiplying the count by a factor of 10 as the membrane filter area is considered equivalent to 100 squares.

5. C. - Procedure Fecal Coliform Analysis:

- a) The MacConkey membrane broth plates for fecal coliform analysis are incubated at $44.5 \pm 0.5^{\circ}\text{C}$ for 20 ± 1 hour. Prolonged incubation may affect the appearance of the colonies. Plates should be counted immediately after removing from the incubation chamber.
- b) Using the stereoscopic microscope at 10X, all yellow, yellow-brown, and yellow-green colonies are counted as fecal coliform colonies. The yellow to brown colour may consist of only a central spot for some colonies, which gradually increases in size for other colonies until the colour covers the entire colony. Those colonies which are blue or gray or colourless should not be counted as fecal coliforms. Doubtful colonies should be confirmed in an EC broth medium, incubated at 44.5°C for 24 hours for evidence of gas production.

- c) An ideal density range for fecal coliform counts would be 20-60 colonies per plate. Plates with greater than 150 fecal coliform colonies or confluent growth represent unsuitable plates for counting and the analysis should be repeated at a more appropriate dilution.

5. D. - Procedure- Fecal Streptococcus Analysis:

- a) The m-Enterococcus agar plates for fecal streptococcus analysis are incubated at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 48 ± 3 hours.
- b) Using the stereoscopic microscope at 10X, all colonies that are red, maroon or pink are counted as fecal streptococci. Colourless, white or yellow colonies are not counted. Doubtful colonies should be confirmed in an Ethyl Violet Azide (EVA) medium incubated at 35°C for 48 hours. Turbid growth in this medium constitutes a positive result.
- c) A suitable density range for fecal streptococci counts would be with plates having 20-100 colonies. Plates with greater than 150 fecal streptococci colonies or confluent growth are considered unsuitable and a higher dilution should be chosen for a repeat analysis, if the sample is not too old.

6. Calculation and Reporting:

The coliform, background, fecal coliform and fecal streptococcus densities of a sample are recorded in terms of numbers of organisms per 100 ml. The general equation for calculating results would be:

$$\begin{array}{lcl} \text{Number of organisms} & & 100 \times \text{colonies counted} \\ \text{per 100 ml} & = & \text{ml sample filtered} \end{array}$$

This calculation would be repeated for each type of indicator organism for which a count was obtained.

7. Preparation of Media for Microbiological Analysis:

I) - The medium for conducting the total coliform and background count analysis is prepared from a dehydrated powder known as Bacto-m Endo Agar LES (Difco). This medium is not autoclaved, therefore, aseptic conditions must be maintained throughout the preparation. Approximate preparation time is one hour.

a) The apparatus required for preparing the medium consists of:

- 1) thermometer (0-100°C)
- 2) two stirring hot plates
- 3) pair of asbestos gloves
- 4) top load balance sensitive to 0.1 g
- 5) bunsen burner
- 6) sterile 10 ml pipettes
- 7) spatula
- 8) one large stirring magnet
- 9) sterile 1 litre graduate cylinder with aluminum foil cover
- 10) large pair of forceps
- 11) sterile 2 litre Erlenmeyer flask with aluminum foil cover
- 12) sterile square plastic petri dishes - 100 x 15 mm (approx. 50-60)
- 13) sterile 1 litre Erlenmeyer flask with screw cap
- 14) one small fan (optimal)
- 15) 100 ml beaker half filled with alcohol

b) The reagents required for preparing the medium include:

- 1) dehydrated m-Endo LES agar (Difco)
- 2) 95% ethyl alcohol
- 3) 100 ml of sterile distilled water in an Erlenmeyer flask

c) Preparation of 1 litre of medium:

- 1) Check the balance to ensure that it is level and operational -zero the balance.
- 2) Place the 2 litre Erlenmeyer flask on the balance and tare the balance to zero.
- 3) Sterilize the clean spatula by dipping it in a 100 ml beaker half-filled with 95% ethyl alcohol and pass the spatula through the bunsen burner flame to burn off the alcohol.
- 4) Weigh out 51 g of the dehydrated m-Endo LES agar powder into the 2 litre flask.
- 5) Cover the mouth of the flask with aluminum foil.

- 6) Flame the mouth of the 1 litre flask containing distilled water and the 1 litre graduate cylinder and measure out 1 litre of distilled water into the screw cap 1 litre flask.
- 7) Using a 10 ml pipette, add 10 ml of 95% ethyl alcohol to the screw cap flask containing the measured 1 litre of sterile distilled water and gently mix the contents.
- 8) Pour about 500 ml of the alcohol-water mixture into the flask containing the powdered medium and swirl the flask to thoroughly wet the medium, ensuring that none of the powdered medium is adhering to the bottom or sides of the flask.
- 9) When the powdered medium is thoroughly wetted and mixed, the remainder of the alcohol-water mixture is added to the flask.
- 10) Using the forceps, the stirring magnet is removed from a beaker of alcohol; flamed; and dropped into the flask.
- 11) The flask is then placed on the stirring hot plate; the stirrer is activated to stir at medium speed; and the heat is turned on full.
- 12) The thermometer is swabbed with alcohol and the temperature of the flask is checked as the temperature rises until a temperature of 94-95°C is reached.
- 13) By the time, the above temperature has been reached, the medium should be totally in solution and be a dark red wine colour. The flask is removed from the hot plate and transferred to the other hot plate which just has the stirring device activated. The fan should be moved into position to assist with the cooling of the medium to about 45-50°C or until the flask can be grasped comfortably in one's hands.
- 14) During the heating process, the 100 x 15 mm petri dishes are laid out along the bench, previously swabbed with dettol. When the medium is cooled to 45-50°C, about 15-20 ml of the medium are poured aseptically into each petri dish and the lid of each dish is left slightly ajar to facilitate solidification of the medium without excess condensation on the lid of each petri dish.
- 15) When the medium has solidified, the lids are closed; the petri dishes are inverted and placed in covered cakettes in a refrigerator at 4°C until required for analysis. The date when the medium was made is marked on each cakette. The plates should be kept in total darkness in the refrigerator at 4-5°C and stored for no longer than 3 days before being used.

- 16) Clean up the preparation area, making sure that the hot plate, stirring mechanisms, and the weighing scale have also been cleaned up and turned off.

II - The medium for conducting fecal coliform analyses is prepared from tablets of Mac Conkey Membrane Broth (Oxoid).

- a) The apparatus required for preparing the medium consists of:

- 1) one 6 oz bottle for each 50 ml of medium
- 2) one 50 ml graduate cylinder
- 3) rack(s) for holding 6 oz bottles during sterilization

- b) The reagents required for preparing the medium include:

- 1) MacConkey Membrane Broth tablets (Oxoid)
- 2) Distilled water

- c) Preparation of medium:

- 1) The dehydrated medium is supplied in tablet form. One tablet is required to prepare 10 ml of medium.
- 2) Place 5 tablets into each 6 oz bottle and using the graduate cylinder measure 51 ml of distilled water into each 6 oz bottle.
- 3) The tablets do not have to be dissolved before the medium is autoclaved.
- 4) The 6 oz bottles are placed in racks and loosely capped for sterilization. The medium is autoclaved at 121°C for 15 minutes.
- 5) After sterilization, the medium is allowed to cool for a few minutes at room temperature and then placed in a refrigerator at 4-5°C to hasten the cooling process. When the medium is completely cooled, the bottle caps are tightened and the medium is kept under refrigeration until used.
- 6) The date of preparation should be written on each bottle, which should preferably be used within one month from this date.

III - The medium for conducting fecal streptococci analysis is prepared from a dehydrated powder form of m-Enterococcus Agar (Difco). This medium is not autoclaved, therefore, aseptic conditions must be maintained throughout the preparation. Approximate preparation time is one hour.

a) The apparatus required for preparing the medium consists of:

- 1) one sterile wide mouth 1 litre Erlenmeyer flask with aluminum foil cover
- 2) one top load balance accurate to 0.1 g
- 3) one bunsen burner
- 4) two stirring hot plates
- 5) weighing boats
- 6) one thermometer (0-100°C)
- 7) pair of asbestos gloves
- 8) one large stirring magnet
- 9) one sterile 500 ml graduate cylinder
- 10) one spatula
- 11) large pair of forceps
- 12) sterile round, plastic petri dishes - 50 x 12 mm (Millipore) (approx. 100-110)
- 13) one sterile Cornwall pipetting syringe assembly, which is set to deliver 4.5 ml of medium
- 14) one small fan
- 15) 100 ml beaker half filled with alcohol

b) The reagents required for preparing the medium include:

- 1) dehydrated m-Enterococcus Agar (Difco)
- 2) 95% ethyl alcohol
- 3) sterile distilled water in 1 litre flask

c) Preparation of 500 ml of medium:

- 1) Check the balance to ensure that it is level and operational -zero the balance.
- 2) Place the weighing boat on the balance and tare the balance to zero.
- 3) Sterilize the spatula by dipping it in alcohol and passing it through the bunsen burner flame.
- 4) Weigh out 21 g of dehydrated m-Enterococcus Agar powder into the weighing boat.

- 5) Using aseptic procedure, remove the aluminum foil cover and flame the mouth of the 1 litre Erlenmeyer flask. Carefully transfer the powder in the weighing boat to this flask and recover it.
- 6) Remove the cap from the sterile distilled water flask, flame the mouth and measure 500 ml into the sterile graduate cylinder.
- 7) Aseptically add the 500 ml of sterile distilled water to the flask containing the powdered medium and gently swirl the flask to ensure that none of the powder medium is adhering to the bottom or sides of the flasks.
- 8) Alcohol flame the large stirring magnet and place it in the flask.
- 9) The flask is then placed on the stirring hot plate; the stirrer is activated to stir at medium speed; and the heat is turned on full.
- 10) The thermometer is swabbed with alcohol and the temperature of the flask is checked as the temperature rises until a temperature of 94-95°C is reached.
- 11) By the time, the flask has reached the above temperature, the medium should be totally in solution and be a clear, straw colour. The flask is removed from the hot plate and transferred to the other hot plate which just has the stirring device activated. The fan should be moved into position to assist with rapid cooling of the medium to about 55°C.
- 12) During the heating process, the 50 x 12 mm petri dishes are laid out along the bench previously swabbed with dettol and the lids pried slightly open.
- 13) The sterile pipetting syringe is unpackaged from its sterile wrappings and the sinker is flamed and aseptically lowered into the m-Enterococcus agar.
- 14) The plunger is then depressed several times to draw the molten medium up into the syringe and the first portion of the medium is wasted to an empty beaker. The medium is then dispensed as quickly as possible in 4.5 ml aliquots into the petri dishes. If bubbles form on the surface of the medium during the pipetting operation, the bunsen burner flame is gently passed over the agar surface several times until the bubbles are removed, while the medium is still in the molten state.

- 15) The petri dishes are left standing until the medium is solidified, after which they are inverted and packed in a Millipore carton or cakette, which is labelled with the name of the medium and preparation date. The petri dishes are stored in the refrigerator at 4°C for a period not exceeding one month.
- 16) Clean up the preparation area, making sure that the hot plates, stirring mechanisms, and the weighing scale have been cleaned up and turned off. The pipetting syringe assembly should be thoroughly washed out in hot water immediately after use and rinsed in distilled water before wrapping in paper for sterilization.
- 17) Every effort should be made to avoid overheating of microbiological media during its preparation.

8. Bibliography

- i) American Public Health Association. 1971. Standard methods for the Examination of Water and Wastewater, 13th ed. Amer. Pub. Health Ass., Inc. New York, N.Y.
- ii) U.S. Environmental Protection Agency. 1975. Handbook for Evaluating Water Bacteriological Laboratories. Second Edition. EPA-670/9-006.
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